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(57) Abstract

A cationic lipid for transfection of macromolecules in which the lipid has a polyether or glyceryl backbone which lipids can be contained in a liposome to effectively transfect a variety of cell types and improve the efficiency of transfection. Compositions containing said lipids and methods of using the same are also disclosed.

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Novel Polycationic Lipids

Related Applications

This application claims the priority of U.S. Application Serial No. 60/098,073 filed on August 27, 1998. The contents of that prior application is fully incorporated herein by reference.

Field of the Invention

This invention relates to polycationic lipids useful for the delivery (transfection) of nucleic acids (DNA,RNA) and other negatively charged or neutral molecules into living cells, either *in vivo* or *in vitro.*

Background of the Invention

Liposome aggregates made with polycationic lipids are useful structures capable of complexing with negatively charged macromolecules such as DNA or RNA. These complexes can be taken up by living cells and then, once inside the cytosol, through an unknown mechanism, they are presumed to migrate into the cell nucleus. In the nucleus, there are enzymes capable of "reading" and "expressing" the message coded by the nucleic acids so delivered and produce new proteins, which were not being produced by the cell before the transfection of the foreign nucleic acid. When cells so transfected divide and their daughter cells still have the capability to produce the proteins encoded by the initially transfected DNA, the transfection is said to be stable. That is, the new DNA has stably integrated into the cell nucleus changing the cell's genetic make-up. If, on the other hand, the parent cells can produce the protein encoded by the transfected DNA, but their daughter cells are not capable of expressing that tranfected DNA, the transfection is said to be transient. RNA transfection is always transient. Stable transfection of human or animal cells is the basis of so called gene therapy, since cells which are deficient in a crucial protein for the organism's survival could in principle be repaired by stably transfecting the DNA needed to produce the absent protein. Another type of potential use of DNA/RNA transfection for therapy is

antisense therapy. In this approach, a short piece of nucleic acid (oligonucleotide) capable of adhering (hybridizing) to defective DNA (or RNA) which is being expressed by the cells to produce an undesired protein, such as an oncoprotein (cancer causing protein), is transfected into the cells in order to stop the expression of the undesired protein by virtue of its adherence to the defective nucleic acid. This method of therapy does not change the genetic make-up of the cell, but blocks the effect of the genetic disorder already present in the cell's genome. Besides these potential applications of polycationic lipids for use in human therapy, there is already a well established market for these types of chemicals in the research products field. They are currently being used by researchers to deliver nucleic acids and proteins into cells in order to study how the expression of different genes affect cell growth and function.

There are two possible ways to deliver DNA into cells for gene therapy : *ex vivo* and *in vivo* transfection. In the *ex vivo* modality, cells from a patient are removed from the body, cultured and transfected *in vitro*. Then, the cells are returned into the patient where the beneficial DNA message is hopefully expressed. In the *in vivo* mode, the DNA is delivered directly into the patient, which makes this procedure simpler and less expensive. To date, the only effective way to deliver DNA *in vivo* is by using a virus which naturally infects cells of a specific organ (targets that organ) within the body, and whose genetic make up has been modified by adding the DNA beneficial to the patient. Once inside the cells of the patient, the virus can incorporate the new DNA in the genome of the cell (stable transfection) and the parent cell and its daughters can express the beneficial protein. The pathological component of the virus has been deleted before the patient is exposed to such a virus and only the targeting component left intact. Virus can do this process sometimes with nearly 100% efficiency. However, there are risks associated with their use, since they can produce immunological reactions which may be fatal to the patient; the DNA incorporation in the cell's genome is random, therefore it might disrupt needed genes or activate oncogenes; they are also difficult to mass produce, and so forth.

Liposomes or lipid aggregates do not have the side effects of viruses, but are not as efficient as viruses are. There is a constant need to develop newer lipids that can approach the efficiency of viruses without their undesirable side effects (E. Marshall, *Science* **269**,1050 (1995)) . There are several lipids for nucleic acids transfection already in the market. The most relevant of these lipids are: (a) DOTMA (N-[1-(2,3-dioleoyloxy)propyl]- N,N,N -

trimethylammonium chloride, U.S. Pat. No. 4,897,355 to D. Eppstein et al.); (b) DMRIE (D,L-1,2-O-dimyristyl-3-dimethylaminopropyl-b-hydroxyethylammoniumchloride, U.S. Pat. No 5,264,618 to Felgner, P.L. et al.); (c) DOTAP (1,2-bis(oleoyloxy)-3-3(trimethylammonia)propane) Boehringer-Mannheim Catalog No.1 202 375); (d) DOGS (5-carboxysperminyglycine dioctadecylamide, U.S. Pat. No 5,171,678 to Behr, J-P. et al. DOGS is sold under the trade name Transfectam™ by the Promega Corp. Madison, WI); (e) DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxyamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate, U.S. Pat. No. 5,334,761 to Gebeyehu, G. et al.); (f) DDAB (Dimethyloctadecylammoniumbromide, U.S. Pat. No. 5,279,833 to Rose, J.K.); and (g) TMTPS (N,N,N,N-Tetramethyltetrapalmylspermine, PCT Int.Pub.No. WO 95/17373. Haces, A. et al.). DOTMA, DOSPA, DDAB and TMTPS are sold by Life Technologies, Inc., Gaithersburg, MD, under the trade names of Lipofectin, LipofectAMINE, LipofectACE and CellFECTIN, respectively. A recent relevant publication which deals with art related to the present invention has been reported by Ruysschaert et al.((1994)*Biochem. Biophys. Res. Commun.*, 203, 1622 -1228). All of these lipids, except DOGS, are formulated with dioleolylphosphatidylethanolamine (DOPE), which is a neutral lipid devoid of transfection activity, in order to make the active liposomes. These lipids possess some desirable characteristics, however they are far from the ideal vehicle to deliver DNA. Their main drawbacks are low efficiency, non-specificity of targeting, considerably toxicity, low water solubility, and serum inhibition of their action.

Although progress has been made in overcoming some of these obstacles, there is considerable room for improvement. The design of these lipids is still a semi-empirical endeavor, since very little is known about the mechanism by which they act.

Therefore, it is an object of this invention to improve the desired characteristics of these lipids by exploring and incorporating new chemical functionalities as well as spatial or topological arrangements which improve the transfection efficiency and lower the toxicity.

It is also an object of this invention to synthesize polycationic lipids which incorporate a small, non lipid-bilayer-disturbing moiety that mimics a natural molecule, which cells can recognize as their natural effector or ligand, thus facilitating the transfection as well as the specificity of targeting of the macromolecule.

Summary of the Invention

In accordance with the invention, a series of new polycationic lipids, their use, and their method of preparation is described. Such lipids are useful as transfection reagents for nucleic acids, oligonucleotides, mononucleotides, polypeptides, and proteins. In addition, some of these lipids are also useful as more effective detergents for cleaning and as vehicles in the cosmetic field.

In one aspect of the present invention, there are described novel oxo and sulfinyl backbone substituted polycationic lipids with ammonium, guanidinium and amidinium positively charged moieties as anchoring groups having Formula I, as shown below and in WO 97/42819 (International Application No. PCT/US97/09093), the contents of which is fully incorporated herein by reference.

The present invention also encompasses a series of novel phosphatidyl and glyceryl guanidinium cationic lipids having the formula 2 shown below and in Figure 8.

In a preferred form of the present invention, the backbone of the substituted polycationic lipis is comprised of a polyether in accordance with Formula 3 as shown in Figure 9:

In a still further aspect of the present invention, there are provided cationic lipids with a glyceryl backbone having Formula 4 as shown below and in Figure 10.

Brief Description of the Drawings

The following drawings are illustrative of embodiments of the invention and are not intended to limit the invention as encompassed by the claims forming part of the application.

Figure 1 is a graphic representation of Scheme I for the preparation of polycationic lipids of the present invention;

Figure 2 is a graphic representation of Scheme II for the preparation of polycationic lipids of the present invention;

Figure 3 is a graphic representation of Scheme III for the preparation of polycationic lipids of the present invention;

Figure 4 is a graphic representation of Scheme IV for the preparation of polycationic lipids of the present invention;

Figure 5 is a graphic representation of Scheme V for the preparation of polycationic

lipids of the present invention;

Figure 6 is a graphic representation of Scheme VI for the preparation of polycationic lipids of the present invention;

Figure 7 is a graphic representation of Formula 1, which represents a series of polycationic lipids of the present invention;

Figure 8 is a graphic representation of Formula 2, which represents a second series of polycationic lipids of the present invention;

Figure 9 is a graphic representation of Formula 3, which represents a third series of polycationic lipids of the present invention; and,

Figure 10 is a graphic representation of Formula 4, which represents a fourth series of polycationic lipids of the present invention.

Figure 11 is a graphic representation of compound 13a of the present invention.

Figure 12 is a graphic representation of compound 13d of the present invention.

Figure 13 is a graphic representation of compound 5a of the present invention.

Figure 14 is a graphic representation of compound 13b of the present invention.

Figure 15 is a graphic representation of compound 6d of the present invention.

Figure 16 is a graphic representation of compound 15 of the present invention.

Detailed Description of the Invention

The compounds of the present invention can be used alone or in mixtures with other liposome forming compounds (co-lipids) to prepare lipid aggregates which are useful to deliver macromolecules, specifically negatively charged macromolecules to living cells either in culture (*in vitro)* or *in vivo*. Colipids are compounds capable of producing stable liposomes, alone, or in combination with other lipid components. Such colipids are preferably neutral, although they can alternatively be positively or negatively charged. Some colipids are disclosed, for example, in U.S. Patent No. 4,897,355, the entire contents of which is incorporated herein by reference. Such examples include phospholipid-related materials, such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine,

phosphatidylserine, phosphatidylinositol, sphinogomyelin, cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPG), dioleoylphosphatidylglycerol (DOPC), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE) and dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (DOPE-mal). Additional non-phosphorous containing lipids are, e.g., stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerol ricinoleate, hexadecyl stereate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide and the like.

Compounds of Formula I:

The lipids depicted in Formula I have a hydrocarbon backbone substituted with heteroatoms which are sterically smaller, but equally or more flexible as the methylene group that they replace. This feature makes these new lipids fit more closely to the macromolecule to be delivered to the cells. This closer fit combined with the polycationic nature of the backbone produces a tighter binding. In addition, these heteroatoms are hydrophilic; thus, they not only confer an increased amphiphilic character to the lipids but also make the backbone more linear or "stretched" as compared to the all-methylene groups backbone. The latter being hydrophobic tends to wrap around itself in an aqueous environment, therefore pulling the positively charged moieties away from the negatively charged phosphates on the DNA/RNA backbone. This results in a weaker binding between the polycationic lipid

backbone and the polyanionic DNA backbone, since the opposite charges can not align properly in this arrangement. The hydrophilic backbone being linear allows for proper alignment of the opposite charges, also leading to a tighter binding. In addition, the higher hydrophilicity conferred by the heteroatoms on the polycationic backbone make possible the addition of more hydrophobic tails without loss of water solubility, thus making these compounds more densely packed than compounds of the prior art. This is an advantage since the same molar amount of lipid will produce a higher hydrophobic coating of the nucleic acid to be delivered. In fact, one of the preferred embodiments is compound 5a, as shown in Figure 13, which has four hydrophobic tails and two positive charges (two tails per charge).

Some of the compounds herein described, such as compounds 13a and 13b (shown in Figures 11 and 14) have fewer hydrophobic tails and a more hydrophobic backbone (no heteroatom substitution, but less hydrophobic overall). However, these compounds have moieties which mimic small natural biological effectors such as the neurotransmitters gamma amino butyric acid (GABA), acetylcholine etc. These moieties bind to their corresponding cell receptors targeting the delivery of nucleic acids to those cells rich in these type of receptors such as muscle and neural cells. The latter type of cells are among the most difficult to transfect since they are postmitotic cells (non-dividing). These small chemical moieties do not perturb the ability of the lipids to form liposomes aggregates and at the same time confer more amphiphilic character to said lipids, since they are polar entities. Particularly preferred embodiments are compound 13a and compound 13d, as shown in Figures 11 and 12.

Another novel feature of the compounds disclosed herein is the fact that, in addition to the traditional quaternary ammonium salts, guanidino and amidino moieties are used as permanent positively charged centers. These functional groups are strongly basic and have

the same charge as their ammonium counterparts, but have the advantage of being sterically smaller, since they are planar. Thus, they can get closer to the negatively charged phosphates of the DNA/ RNA backbone producing a stronger binding interaction than that of ammonium salts. Furthermore, these guanidinium and amidinium moieties have the ability to form hydrogen bridges with the nucleic acids' bases (guanidinium salts are used as chaotropic agents to precipitate DNA) therefore they have an additional binding mode not available to ammonium salts. Moreover, the guanidino moiety can also be used to target neural cells, since compounds such as Guanethidine, which possess such a functional group, are internalized by neurons (Wiener, N. In, *The Pharmacological Basis of Therapeutics*, Gilman, A.G.; Goodman,L.S.; Rall, T.W.; and Murad, F.; Eds. Macmillan Pubs. Co. New York, **1985**, pp. 181-214.) . Thus, by including the lipidic content as well as the amine and guanidino moieties of Guanethidine in our novel liposome reagents we can target this difficult to transfect cell type. A particularly useful and preferred embodiment of these compounds is compound 6d, as shown in Figure 15, which is the most active of the compounds tested. Additionally, reduced or no toxicity was observed for these lipids at the concentrations tested.

Compounds of Formula II:

Despite all the reasons given above in order to "rationally design" these lipids, it is still impossible to predict their DNA transfection activity at this time. In fact, cationic lipids for DNA transfection already in the market such DOTAP, DOTMA, DMRIE and DORI whose chemical structures are almost identical to that of the cationic lipid known as the Rosenthal Inhibitor (Rosenthal, A.F. and Geyer, R.P., *J. Biol. Chem.* 235(8):2202 (1960)) have significant transfection activity, unlike the Rosenthal Inhibitor which is reported to be inactive as a DNA

transfection reagent (see U.S. Pat No 5,264,618 to Felgner, P.L. et al.). The formulae for some prior known compounds, as shown in WO 97/42819 (International Publication Date 20 November 1997) at the top of page 9, illustrate this point more clearly.

Compounds of formula II described herein also have a similar structure to that of the Rosenthal Inhibitor. However, these compounds differ from the Rosenthal Inhibitor in that a guanidinium or amidinium functionality is used as the positively charged anchoring group, and they also lack a quaternary ammonium group at the C-1 position of the glycerol backbone. A preferred embodiment of these latter type of transfection reagents is compound 15, as shown in Figure 16.

An interesting feature of this compound is that it has the ability to form liposomes without the need of co-adjuvants such as DOPE or DOPC. Thus, it can be used to form liposomes with other cationic lipid compounds.

Specific Examples of the Invention.

Scheme I

Scheme I, as shown in Figure 1, shows the general synthetic route to prepare polycationic lipids having a heteroatom substituted anchoring backbone. Thus, diglycolyl chloride (1) was treated with a suitable primary or secondary amine (2a-d) in methylene chloride in the presence of a base such as triethyl amine under an inert gas such as argon at room temperature to obtain the corresponding diglycolamides (3a-d). These amides were reduced with lithium aluminum hydride (LAH) or can be reduced with borane in refluxing anhydrous tetrahydrofurane (THF) to afford the corresponding amines (4a-d). Secondary amine (4c) was easily converted to the corresponding tertiary amine (4e) upon treatment with

acrylonitrile. Compound (4e) can be treated with ammonium chloride at high temperature to produce the corresponding amidine(6e). Alternatively, the latter amidine derivatives can be obtained by reacting the dinitrile (4e) with anhydrous hydrogen chloride in ethanol, followed by treatment of the imidoester so obtained with ammonium hydroxide. Primary amine (4d) was converted to the target compound (6d) by treatment with S-methyl isothiouronium hydroiodide (S-methyl thiourea) in tetrahydrofurane in the presence of triethylamine. Additionally, this guanidinium derivative can be alkylated with, for example, iodomethane to produce the corresponding quaternary ammonium salt. Tertiary amines (4a,b,e) were treated with an alkylating agent such as iodomethane, iodoethyl acetate or 2-bromo ethyl acetate to afford the quaternary ammonium salts (5a,b,f). The latter compounds were also synthesized by treating the corresponding tertiary amines with the commercially available 2-bromoethyl ether (lower panel, scheme I). This route has only two steps, but is not as flexible or prolific as the route depicted in the upper panel of Scheme I.

Scheme II

Compound (7) was readily synthesized, as shown in Figure 2, by treating commercially available 1,4-diaminobutane with acrylonitrile. Diamide-dinitrile (8) was then easily obtained by treatment of compound (7) with an acyl halide such as palmitoyl chloride in methylene chloride in the presence of triethyl amine. The diamide-dinitrile (8) was reduced with lithium aluminum hydride or can be reduced with borane in THF to the corresponding tertiary and primary amines functionalities to afford compound (9). Guanidinium compound (10) can be obtained in a similar fashion as shown in Scheme I for compound (6d) by reacting the primary amines of compound (9) with S-methyl thiourea in THF and triethylamine.

Scheme III

Referring to Figure 3, tetrapalmyl spermine (11) (Haces, A. et al. PCT Int. Pub. No WO 95/17373) was treated with ethyl iodoacetate at room temperature to afford the tetraalkylated derivative (13a). Similarly, compound (11) was treated with 2-bromoethyl acetate at high temperature to afford (13b). Reaction of (11) with 4-bromo or 4- chloro butyryl chloride in methylene chloride in the presence of triethyl amine at low temperature gave the corresponding 4-bromobutyramide derivative (12). Intermediate (12) was immediately treated with iodomethane to produce the N,'N''- dimethylated intermediate (13c) which in turn was treated with an excess of ammonium hydroxide in tetrahydrofurane at elevated temperature to convert the bromide (or chloride) into the corresponding primary amine (13d). All of these compounds have moieties which resemble or mimic the neurotransmitters gamma aminobutyric acid (GABA, compound (13d)) and acetylcholine (compounds (13a,b)). These small groups do not change substantially the liposomal forming ability of the lipid molecule and at the same time are capable of being recognized by neural or muscles cells. This preferential recognition by these type of cells makes these lipids target specific DNA/RNA delivery agents.

Scheme IV

Referring to Figure 4, commercially available dioleoylphosphatidylethanolamine (14) was treated with an excess of S-methyl isothiouronium hydroiodide in tetrahydrofurane and in the presence of triethylamine to afford the corresponding guanidinium compound (15).

EXAMPLES

Example 1: Synthesis of octadecylcyanoethylamine (2d).

Octadecylamine (2g, 7.4 mmol) and acrylonitrile (15ml) were heated for 3h at 70°C in a thick wall test tube capped with a teflon lined cap. TLC (silica gel; ethyl acetate) shows a new spot at rf = 0.65 . The excess acrylonitrile was removed in vacuo to afford pure product (2.40g, 100% yield). H-NMR ($CDCl_3$) : δ0.88 (t, 3H), 2.25 (br.s.,32H), 2.53 (t, 2H), 2.62 (t,2H), 2.93 (t,2H). FT-IR (cm^{-1}) 2250 (CN).

Example 2: Synthesis of bis (mono and dialkyl)diglycolamides(3a-d), general procedure.

To a solution of dialkylamine (2mmol) and triethylamine (2 mmol) in methylene chloride (250ml) under argon was added diglycolyl chloride (1mmol) and the resulting solution was stirred for 18h at room temperature. TLC (silica gel; MeOH or CH_2CL_2/THF,3:1) shows the absence of starting material and a new spot. The methylene chloride solution was washed with sodium bicarbonate (10% in water), dried (Na_2SO_4) and the solvent removed, to afford the desired diamide.

Proceeding as described before and using the appropriate mono or dialkylamine the following compounds were prepared:

3a. Bis (dioctadecyl) diglycolamide (81% yield), H-NMR (CDCL3) δ: 0.88 (t,12H), 1.25 (s, 124H), 1.5 (br.s, 8H), 3.15(t,4H), 2.9 (t,4H), 3.2 (s, 4H). R (cm^{-1}): 1651 (C=O);

3b. Bis (didecyl)diglycolamide (73% yield), H-NMR(CDCL3) δ X: 0.85 (t,12H), 1.25 (s,

64H), 1.5 (br.s,8H), 3.18 (t,4H), 3.3 (t,4H), 4.4 (s, 4H). FT-IR(cm^{-1}): 1657(C=O);

3c. Bis (octadecyl)diglycolamide (100% yield),H- NMR(CDCL3) δ: 0.85 (t, 6H), 1.25 (br.s, 60H), 1.5 (br.s,4H), 3.3 (q,4H), 4.05(s,4H), 6.4 (br.s,2H);

3d. Bis (octadecylcyanoethyl) diglycolamide (98%yield), H-NMR ($CDCl_3$) δ : 0.85 (br.t, 6H), 1.1-1.6 (br.s, 64H), 2.65 (t, 4H), 3.25 (br.t, 4H), 3.55 (br.t, 4H), 4.3 (s, 4H);

Example 3: Synthesis of N,N,N',N'-Mono and dialkyl 2,2'-oxybis ethylamines (4a-d), general procedure

To a solution of lithium aluminum hydride (6 to 64 molar excess) in dry tetrahydrofurane (THF) was added the corresponding diamide in small portions under a blanket of argon. The resulting mixture was refluxed for two to three days under argon. The progress of the reaction was followed by TLC (silica gel; CH_2Cl_2/TFH, 3:1 for dialkylamides; 10% triethylamine in CH_2Cl_2 for monoalkylamides and triethylamine for cyanoethylamides). The reactions were quenched with sodium hydroxide (10% in water). The mixture was filtered, the filtrate dried (Na_2SO_4), and the solvent removed in vacuo to afford the desired products.

Proceeding as described above and using the appropriate diamide the following compounds were obtained:

4a. N,N,N',N'-dioctadecyl-2,2'-oxobis ethylamine (77% yield), H-NMR($CDCl_3$) δ: 0.88 (t, 12H), 1.25 and 1.43 (br.s., 128H), 2.42 (t, 4H), 2.62 (t, 4H), 3.48 (t, 4H), 3.7, (t,4H);

4b. N,N,N',N'-didecyl-2,2'-oxobis ethylamine (93% yield), H-NMR ($CDCL_3$) δ: 0.88 (t,12H), 1.25 and 1.42 (br.s, 64H), 2.42 (t, 8H), 2.63 (t, 4H), 3.5 (t, 4H);

4c. N,N',-octadecyl-2,2'-oxobis ethylamine (71% yield), H-NMR ($CDCl_3$) δ: 0.87 (t, 6H), 1.25 and 1.45 (br.s., 64H), 2.6 (t,4H), 2.78 (t, 4H), 3.65 (t,4H);

4d. N,N,N',N'-octadecylaminopropyl-2,2'-oxobis ethylamine (80% yield), H-NMR (CDCL3) δ:0.88 (t,6H), 1.25 and 1.45 (br.s,70H), 2.4-2.8 (br.m,16H), 3.4-3.7 (br.m, 4H).

Example 4: Synthesis of N,N,N,N',N',N' - dioctadecylmethyl-2,2'-oxybisethylammonium iodide.(5a).

N,N,N',N'-dioctadecyl-2,2'-oxy bis ethylamine (19mg, 0.017mmol) was dissolved in iodomethane (1ml) inside a capped thick-wall test tube, and the resulting solution heated for 20h at 75°C . TLC (silicagel ; chloroform:acetone:methanol:water; 50:15:5:5:1) shows only one spot at Rf=0.8 , which gives a negative ninhydrin test and no starting material. The excess iodomethane was removed in vacuo to afford the desired product (23 mg, 96%). H-NMR($CDCl_3$) δ: 0.88 (t,12H), 1.15 - 1.5 and 1.7 (br.s.,128H), 3.35 (s, 6H), 3.46 (br.m.,8H), 3.88 (br.s.,4H), 4.28 (br.s., 4H). Proceeding in a similar fashion as per compound (5a) , compound (5b) was obtained in 100% yield.

Example 5: Synthesis of N,N,N',N'-cyanoethyloctadecyl-2,2'-oxybis ethylamine (4e).

A suspension of N,N'-octadecyl-2,2'-oxybis ethylamine (100mg,0.16mmol) in acrylonitrile (4ml) was heated for 18h at 80°C in a capped, thick-wall test tube (the initially

liquid two phase system became a clear homogenous solution after 2h). TLC (silicagel;dichloromethane/ THF, 3:1) shows the absence of starting material and a spot corresponding to desired material at rf = 0.95. The excess acrylonitrile was removed in vacuo to afford the desired material. H-NMR($CDCl_3$) δ: 0.88 (t, 6H), 1.25 (br.s., 64H), 2.45 (2t,8H), 2.68 (t, 4H), 2.87 (t,4H), 3.5 (t, 4H).

Example 6: Synthesis of N,N,N,N',N',N'-cyanoethyloctadecylmethyl-2,2'-oxybis ethyl ammonium iodide (5e).

A solution of N,N,N',N'-cyanoethyloctadecyl-2,2'-oxybis ethylamine (60 mg,0.08 mmol) in iodomethane (1.5ml) was heated for 3h at 80 ^{o}C in a capped, thick wall tested tube. The excess iodomethane was removed in vacuo to afford desired product. H-NMR ($CDCl_3$) δ: 0.88 (t, 6H), 1.25 (br.S., 60H), 1.8 (br.s., 4H), 3.45 (br.s., 6H), 3.65 (br.s., 4H), 3.95-4.4 (br.m., 16H).

Example 7: Synthesis of N,N,N,N',N',N'- acetoxyethyldioctadecyl-2,2'-oxybis ethyl ammonium iodide(5f).

A solution of N,N,N',N'-dioctadecyl-2,2'- oxybis ethylamine (16mg, 14.3 mmol) and ethyl iodoacetate (0.5ml) in chloroform (1ml) was heated for 18h at 75 ^{o}C in a capped thick wall test tube. The chloroform was removed in vacuo and the residue dissolved in tetrahydrofurane (10ml). To this solution was added thiourea and the mixture stirred at room temperature until no more thiourea went into solution (stoichiometric excess after all iodide is converted to the isothiouronium salt). The mixture was heated for 2h at 70^{o}C and the excess solvent removed in vacuo. The residue was then redissolved in dichloromethane (10ml) and the solution washed with water (4x 5 ml), dried (Na_2SO_4), filtered and the solvent

removed in vacuo to afford 10 mg of desired product.

Example 8: Synthesis of S-methylisothiouronium hydroiodide.

A solution of thiourea (0.8g,10.5mmol) and iodomethane (8.8g,62mmol) in methanol (25ml) was heated in a capped thick-wall test tube for 4.5h at 50°C. The reaction mixture was rotaevaporated to afford pure desired material in 100% yield. H-NMR (CD_3OD) δ: 2.62(s, 3H), 4.8 (br.s., 4H).

Example 9: Synthesis of N,N,N',N'-guanidinopropyloctadecyl oxy bis-2,2'-ethylamine hydroiodide (6d).

A solution of N,N,N',N'-aminopropyloctadecyl-2,2'- oxybis ethylamine (100mg, 0.14 mmol) , S- methyl isothiouronium hydroiodide (300mg, 1.3 mmol) and triethylamine (300mg, 3 mmol) in tetrahydrofurane (10ml) were heated in an argon flushed, capped thick-wall test tube for 20h at 95°C. The solvent and methyl mercaptan byproduct were removed in vacuo in a chemical fumes hood and the residue dissolved in methylene chloride (30ml), the organic phase was washed with brine (3x, 20 ml), water (2x 10ml), dried (Na_2SO_4), filtered and the solvent removed to obtain a reddish solid (100mg,80%). H-NMR($CDCl_3$) δ: 0.86 (t,6H), 1.1-1.6 (br.s., 64H), 2.4-2.8 (br.m., 16H), 3.15-3.7 (br.m., 12H). FTIR (cm^{-1}): 1653 (C=NH).

Example 10: Synthesis of N,N'-cyanoethyl-1,4-diaminobutane (7).

1,4-diaminobutane (2g, 22 mmol) was cooled to 0C (ice bath) and to this solid was added acrylonitrile (4ml) . The mixture was let reach room temperature slowly (ca 30min) and then let react for additional 18h at room temperature with stirring. The excess acrylonitrile

was removed in vacuo to afford the desired product. H-NMR ($CDCl_3$) δ: 1.3 (br.s.,2H), 1.5 (br.s, 4H), 2.5 (t, 4H), 2.65 (br.m.,4H), 3.9 (t,4H). FTIR (cm^{-1}) : 2247 (C=N).

Example 11: Synthesis of N,N,N',N'-cyanoethylpamitoyl-1,4-diaminobutane(8).

To a solution of N,N'-cyanoethyl-1,4-diaminobutane (0.714g, 3.68mmol) and triethylamine (0.744g, 7.36mmol) in dichloromethane (150ml) was slowly added palmitoyl chloride (2.02g, 7.36 mmol) and the resulting mixture let react at room temperature overnight. The reaction was washed with sodium bicarbonate (10%, 2x 50ml), water (2x 50ml), dried (Na_2SO_4) , filtered and the solvent removed in vacuo to afford the desired product (2.3g, 93 %). H-NMR ($CDCL_3$) δ: 1.9 (t,6H), 1.25 and 1.6 (br.s., 56H), 2.32 (m,4H), 2.72 (t, 4H), 3.42 (m, 4H), 3.55 (t,4H). FTIR (cm^{-1}) 1643 (C=O).

Example 12: Synthesis of N,N,N',N'-aminopropylpalmyl-1,4-diaminobutane (9).

To a solution of lithium aluminum hydride (600mg, 15.9 mmol) in tetrahydrofurane (50ml) was added N,N,N',N'-cyanoethylpalmitoyl-1,4-diaminobutane (300mg, 0.45 mmol) and the reaction mixture was refluxed for 72 h. Then, a procedure essentially the same as per example 3 (supra) was followed to afford desired diamine (200mg,70%). H-NMR($CDCl_3$) δ: 0.88 (t,6H), 1.25 and 1.5 (br.s, 64H), 2.3-2.7 (br.m, overlap. t, 16H).

Example 13: Synthesis of N,N,N',N'-guanidinopropylpalmyl-1,4-diaminobutane (10).

A procedure identical as per example 6 (supra) was followed. H-NMR ($CDCl_3$) δ: 0.9 (t, 6H), 1.2-1.35 (br.s., 68H), 1.5-2.0 (br.m., 4H), 2.65-3.5 (m, 24H). FTIR (cm^{-1}) : 1650 (C=N).

Example 14: Synthesis of N,N'''-4-bromobutyryl-N,N',N''N'''-tetrapalmylspermine (12).

To a cooled (0 °C) solution of N,N',N'',N'''-tetrapalmylspermine (400mg, 0.36 mmol) and triethylamine (80mg, 0.8 mmol) in dichloromethane (14ml) was added 4-bromobutyryl chloride (156mg, 0.8 mmol) and the resulting mixture was allowed to react for 1h at 0°C. The reaction was quenched and washed with cold sodium bicarbonate solution (10 % in water, 3x 5ml), dried (sodium sulfate), filtered and the solvent removed at room temperature in vacuo to afford a white foam. H-NMR ($CDCl_3$) δ: 0.88 (t, 12H), 1.15-1.5 (br.s., 136H), 2.12 (t, 4H), 2.3-2.55 (br.m.,12H), 3.2-3.36 (br.m., 8H), 3.62 (t, 4H).

Example 15: Synthesis of N,N',N",N'''-tetrapalmyltetraacetoxyethylspermine iodide salt(13a):

A solution of tetrapalmylspermine (Haces et al.,PCT Int. Pub. No WO/95/17373), 130mg, mmol) in neat ethyl iodoacetate (1.5ml) was heated to 75°C for 18h. The reaction was worked up following essentially the same procedure as per example 13 (supra) to afford the desired product.

Example 16: Synthesis of N,N'''-4-bromobutyryl-N,N',N",N'''-tetrapalmyl-N',N"-dimethylspermine(13c).

N,N'''-4-bromobutyryl-N,N',N",N'''-tetrapalmylspermine (350mg,0.25 mmol) was dissolved in iodomethane (3ml) and the resulting solution let react for 2 days at room temperature. Excess iodomethane was evaporated to afford desired product, which is negative for ninhydrin test. H-NMR ($CDCl_3$) δ: 0.88 (t, 12H), 3.41 (br.s., 6H).

Example 17: Synthesis of N,N'''-4-aminobutyryl-N,N',N",N'''-tetrapalmyl-N',N"-

dimethylspermine(13d).

To a solution of N,N'''-4-bromobutyryl-N,N',N",N'''-tetrapalmyl-N',N"-dimethylspermine (100mg,0.05 mmol) in tetrahydrofurane (10ml) was added ammonium hydroxide (20 ml, 28% by weight) and the resulting mixture heated to 70 ^{o}C for 2 days in a capped, thick wall reaction tube. The solvent was azeotropically evaporated (ethanol) to afford a brown solid which is strongly positive for ninhydrin test. H-NMR ($CDCl_3$) δ: 0.88 (t,12H), 1.1-1.4 (br.s,130H),1.5-2.2 (br.m,16H), 3.0-3.8 (br.m,10H), 3.4 (br.s, 6H).

Example 18: Synthesis of dioleoylphosphatidyl ethanolguanidine (15).

To a solution of dioleolylphosphatidyl ethanoamine (70mg, 0.094 mmol) and triethylamine (1ml) in tetrahydrofurane (10 ml) was added S-methylisothiouronium hydroiodide (70mg, 0.32 mmol) and the resulting solution was heated for 18h at 70 ^{o}C. The solvent was removed in vacuo and the residue redissolved in dichloromethane (25ml). This solution was washed with water (2 x 10 ml), dried (sodium sulfate), filtered and the solvent evaporated to afford the desired product (40 mg, 47%). H-NMR ($CDCl_3$) δ 0.88 (t,6H), 1.2-1.44 (br.s, 40H), 2.00 (br.d,10H), 2.29 (t, 4H), 3.19 (q,1H), 3.42 (br.s,1H), 3.85-4.20 (br.m,2H), 4.38 (br.m,1H), 5.3 (br.d, 4H). FTIR (cm^{-1}) : 2361, 1741.

Example 19: Liposomes formulation:

Lipids were formulated by mixing appropriate molar amounts of the active lipid (compounds 5a, 6d and 13d as shown in Tables I and II) with a colipid dioleoylphosphatidyl ethanolamine (DOPE) in dichloromethane and dispersing this mixture in the final amount of water using the solvent vaporization method. (David W. Deamer, in Liposome Technology,

vol.I, p-29, CRC press Boca Raton, Fl, 1984).

Cell culture and plasmids

Cell lines were from the American Type Culture Collection (Rockville, Maryland) and were cultured in RPMI11649, 10% FCS, pen/strep . Plasmid pCMV â-gal, which contains the *E. Coli* â-galactosidase (gene) under the control of the powerful cytomegalovirus promoter (McGregor et al. (1989) *Nucleic Acids Res.*, 17: 2365) was purchased from Clontech, Inc. Primary cells were from human tracheal isolates and neonatal foreskin.

Example 20: Transfection of HepG2 and HeLa cells.

Cells were plated in 48-well plates ($1cm^2$) at a concentration of $1x\ 10^5$ cells/well in 0.5 ml of RPMI-1640, 10% FCS, Pen/step. The next day, lipids aliquats (1,3 and 5μl of 1 mg/ml liposome in water) were diluted in polystyrene tubes containing 100μl of serum-free, antibiotic-free RPMI-1640 and to these tubes were added 150 ng of plasmid in 100μl of the same medium (suboptimal amount in polypropylene tubes) and incubate for 15 min. The cells were washed twice with Dulbecco's PBS, the lipid:plasmid complexes added to them and then incubated for 7h at 37 C^o in 5% CO_2 atmosphere. Growth medium was added to the cells for a final volume of 1 ml and a final concentration of 10% FCS, pen/strep, 50μg/ml gentamicin in RPMI-1640 and were incubated overnight. The results are shown in Tables I and II and discussed hereinafter.

Example 21: Transfection of Primary Human Tracheobronchial and Epidermal Keratinocytes.

Cells were grown in serum free medium (SFM) and plated on 35 mm plates (6wells) such that the confluence after 24h was above 50%. Plasmid reporter (2 μg and 5 μg, respectively) was mixed with variable amounts of liposomes (see tables IV and V) and the complex formed added to the cells. The cells were transfected during 4h and 6h, respectively. The DNA/liposome complex was removed by rinsing with SFM and the cells incubated for 48h under normal growth conditions and then assayed for the appropiate marker.

Example 22: Transfection and CAT assay of Jurkat Cells (suspension cells).

The cell suspension culture was transferred to a 50 ml conical tube and centrifuged at 400g for 10 min. The cells were washed twice by aspirating off the supernatant and gently resuspending the cell pellet in 25 ml of sterile PBS and centrifuging again at 400g for 10min. The pellet was resuspended in a volume of serum-free growth medium such that a final concentration of 6.25 x 10^6 cells /ml was obtained (about 10 ml). 35 mm cell culture plates were inoculated with 0.8 ml of the cell suspension. For each well, 10 μg of CAT plasmid were dissolved in 110 μl of serum-free medium and a separately in another tube were diluted 30 μl of the lipid solution in 70 μl of serum-free medium. The plasmid and lipid solution were mixed and gently swirled and allowed to stand at room temperature for 10 min. The complex DNA/lipid solution was then randomly dropped over the culture well. The wells were gently swirled and then incubated at 37 ^{0}C under a 7 % CO_2 atmosphere for 5 hours. After 5h incubation, 4ml of 12.5 % FBS growth medium were added to the wells and the incubation continued for additional 72 h under the above conditions. The cells were then transferred to 10 ml Falcon tubes and the wells rinsed with 5ml of sterile PBS. The cell suspension was washed twice with 5ml of sterile PBS as previously. The final pellet was

resuspended in 400 μl of lysis buffer and transferred to 1.5 ml centrifugation tubes. The tubes were capped and placed horizontally on a rocker and the cells lysated for 30 min.

100 μl aliquots were then assayed for CAT activity following the procedure of Neumann et al. (1987) *Biotechniques* 5: 444. The results are shown in Table III and discussed hereinafter.

Example 23: Assay for transient transfection (adherent cells).

The cells were washed twice with Dulbecco's PBS and stained with freshly prepared fixative (2% formaldehyde/ 0.2% glutaraldehyde in PBS) for 5 min, washed twice with Dulbecco PBS). Then, the cells were stained with 0.5 ml of β-galactosidase histochemical stain (0.1% x-gal, 5mM potassium ferrocyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$ in PBS) for 24h at 37 C^o in a 5% CO_2 atmosphere. Blue cells (β - gal positive) were counted.

Example 24: Results and Discussion.

The results are summarized in Tables I , II, III, IV and V. Tables I and II show the relative transfection efficiency of compounds 5a, 6d and 13d versus control compound TMTPS (Compound 3 in PCT Int. Pub. No. WO95/17373. Haces, A. et al.) in HepG2 (human hepatocarcinoma) and HeLaS3 (human cervical carcinoma) cells, respectively, and under suboptimal conditions for activity. In these cell lines, compounds 6d and 13d show a 2-2.4 fold higher efficiency than the TMTPS control, and compound 5a is half as active as the control in HepG2 cells and showed negligible activity in HeLaS3 cells. Table III shows an analogous comparison using Jurkat cells (T-cell leukemia). In this experiment, compounds 5a and 13d show similar efficiency as TMTPS, but compound 6d shows almost 38% more

activity than that of the control. Tables IV and V shows the relative efficiency of compounds 6d and 13d in the primary human tracheobronchial epithelial and human keratinocytes cells. Primary cells are cells that are freshly isolated from humans or animals and which, unlike the cultured cell lines, reflect the potential behavior of a compound in vivo more closely. Thus, for genetic therapy to work, it is necessary to be able to transfect these types of cell lines before any in vivo experiments are tried. These types of cells are also the most difficult to transfect and their transfection efficiencies are usually below 1%. Table IV shows the relative efficiency of compounds 6d and 13d versus DOTMA (Lipofectin™ Reagent, Life Technologies, Inc., supra) in primary human tracheobrochial cells. Both of these compounds show a relative range of activities of 5.3 to 6.0 times higher than that of the Lipofectin control. At the same time their cell toxicity was below 5%, unlike the control which showed toxicity in the 10-20% range. Thus, these lipid reagents are superior to the commercial standards in both respects. In addition, this is a very significant result since tracheobronchial cells are involved in the genetic disease cystic fibrosis. There are several genetic therapy clinical trials being conducted at the present time targeting these cells using either viral or liposomal vectors (see 10th Annual North America Cystic Fibrosis Conference, Orlando, Fl., Oct 24-27 (1996), Abstracts or *Pediatr Pulmonol* Suppl, 13: 74-365, Sept, 1996) .

Table V depicts the the percentages of β-gal positive cells (absolute number) which were obtained in primary human epidermal keratinocytes with compounds 5a and 6d versus that obtained with DOSPA control (Lipofectamine™ Reagent, Life Technologies, Inc. supra). Compounds 5a and 6d gave, respectively, 35% and 50% positive cells as compared with 2% positives for the control. This represents a 15-25 fold better efficiency for these novel liposome

reagents when compared with this well known standard. Moreover, primary human keratinocytes are also a potential target cells for genetic therapy (Fenjves, E.S. et al., *Hum Gene Ther* **5**: 10,1241-8, Oct. 1994), but its use has been restricted due to the lack of highly efficient transfection vectors.

TABLE I

TRANSFECTION OF HEP G2 CELLS

Lipid/DOPE (molar ratio)	Optimal Liposome Amount (μg)	β- Gal Positive Cells (%)
Compound 5a (1: 1.8)	3 μg	0.8
Compound 6d (1: 1.5)	3 μg	4.3
Compound 13d (1:1.5)	3 μg	2.9
Control TMTPS/DOPE (1:1.5)	5 μg	1.5

Cells were plated in 48 well plates at a density of 1 x 10^5 per well in 0.5 ml of growth medium. After 24h, the cells were washed with serum free medium and transfected with a suboptimal amount (150 ng) of plasmid pCMV-βgal. using 1,3 and 5μl (1,3 and 5μg) of lipid formulation. The amount giving the highest level of transfection efficiency is shown. The experiment was run in triplicate.

TABLE II
TRANSFECTION OF He La S3 CELLS

Lipid/DOPE (molar ratio)	Optimal Liposome Amount (μg)	β Gal Positive Cells (%)
Compound 5a (1:1.8)	5 μg	0.001
Compound 6d (1:1.5)	3 μg	2.4
Compound 13d (1:1.5)	1μg	2.1

Control TMTPS/DOPE (1:1.5)	5 μg	0.9

Cells were plated in 48 well plates at a density of 1 x 10^5 per well in 0.5 ml of growth medium. After 24h, the cells were washed with serum free medium and transfected with a suboptimal amount (150 ng) of plasmid pCMV- βgal. using 1,3 and 5μl (1,3 and 5μg) of lipid formulation. The amount giving the highest level of transfection efficiency is shown. The experiment was run in triplicate.

TABLE III
TRANSFECTION OF JURKAT CELLS

Lipid/DOPE (molar ratio)	Optimal Liposome Amount (μg)	CAT Activity (mU/well)
Compound 5a (1:1.8)	30 μg	196.00
Compound 6d (1:1.5)	30 μg	298.20
Compound 13d(1:1.5)	30 μg	220.00
Control TMTPS/DOPE(1:1.5)	30 μg	216.44

Wells were inoculated with 6.25 x 10^6 cells. 10 μg of CAT plasmid were mixed with 30μg (optimal amount known for the control) of the lipids and then added to the cells. After 5h the transfection was quenched with FBS containing medium and the cells incubated for 72h. Cells were lysated in 400 μl of buffer. 100 μl aliquots were assayed for CAT activity.

TABLE IV
TRANSFECTION OF PRIMARY HUMAN TRACHEOBRONCHIAL EPITHELIAL CELLS

Lipid/DOPE (molar ratio)	Optimal Liposome Amount (μg)	Luciferase Activity (counts)
Compound 6d (1:1.5)	12 μg	7297022
Compound 13d (1:1.5)	12 μg	8343975

Control , Lipofectin™ *	6 μg	1379341

35 mm plates were inoculated with cell isolates and transfected at 90% confluence. 2μg of firefly luciferase plasmid were mixed with 12 μg of the lipids and then added to the cells. After 5h, the transfection was quenched by removal of the DNA/Lipid complex and the cells incubated for 72h. Cells were lysated and aliquats assayed for luciferase activity. Cell toxicity, determined by the trypan blue method, was below 5% for lipids 6d and 13d and between 10-20% for Lipofectin.

* Lipofectamine was also run as a control, but its efficiency was negligible.

TABLE V
TRANSFECTION OF PRIMARY HUMAN EPIDERMAL KERATINOCYTES

Lipid/DOPE (molar ratio)	**Optimal Liposome Amount (μg)**	β-Gal Positive Cells (%)
Compound 5a (1:1.5)	40 μg	35 %
Compound 6d (1:1.5)	20 μg	50 %
Control , Lipofectamine™	25 μg	2 %

Cells were seeded at $2x10^5$ /well in 35 mm wells and transfected the next day. 5μg of βgal DNA were mixed with the appropiate amount of lipids and added to the cells. After 4h, the medium was replaced and the cells incubated for additional 48h and then assayed. Blue cells were observed under the microscope and counted.

Polyether Backbone Embodiments

In a further embodiment of the present invention, another series of lipids are provided, as disclosed herein. These lipids have a polyether backbone which generally makes them more prone to line-up with the negatively charged polyphosphate backbone of nucleic acids. In addition, the polyether functionality, which is also present in polyethylene glycol (PEG), a known cell-fusogenic agent, may contribute to the

transfection activity of these novel polycationic lipids.

The present lipids have shown to be very effective transfection reagents with cultured cells, especially with the very useful COS and CHO cell lines. These reagents also work with other cells lines such as NIH3T3. The synthesis of a preferred polycationic lipid having a polyether backbone is described below.

Example 25

Synthesis of triethyleneglycoldipalmitoyldiamide [compound 21]

To a solution of triethylene glycol diamine (1g, 6.74 mmol) (compound 20) and triethylamine (1.4g, 13.81 mmol) in dichloromethane (400ml) at room temperature under argon was added (dropwise) palmitoyl chloride (3.79g, 13.81mmol) and the resulting mixture stirred overnight at room temperature. Since the product is partly soluble in dichloromethane, but soluble in chloroform, the dichloromethane was removed in vacuo to about one fifth of the initial volume and chloroform (200ml) was added. The organic layer was washed with 10 % aqueous sodium bicarbonate solution (3x, 50 ml), dried (Na2SO4), filtered, and the solvent removed in vacuo to afford the desired diamide (3.9, 92%).

Synthesis of N,N=-dipalmityl-triethyleneglycoldiamine [compound 22]

A solution of triethyleneglycoldipalmitoyldiamide (1g, 1.6mmol) and lithium aluminun hydride (0.3 g, 8.0 mmol) in dry tetrahydrofurane (200ml) was refluxed for three days under argon. The cooled reaction mixture was treated with 5% sodium hydroxide solution to destroy the excess lithium aluminun hydride. The reaction mixture was then

filtered, dried (Na2SO4) and the solvent removed to afford pure product (0.8g, 84%).

Synthesis of N,N,N=,N=-dimethyldipalmityl-triethyleneglycoldiamine [compound 23]

N,N=-dipalmityl-triethyleneglycoldiamine (240mg, 0.4mmol) was dissolved in an excess of iodomethane (3ml) and the solution allowed to react for one day at room temperature in the dark. The unreacted iodomethane was removed under vaccum and the residue dissolved in ethanol (3ml). To this solution was added a solution of potassium hydroxide (3ml,saturated) in ethanol. The red residue solution turned clear and colorless. The new solution was diluted with dichloromethane (50ml) and washed immediately with aqueous sodium bicarbonate solution (3x 20ml). The organic layer was dried ($NaSO_4$) and the solvent removed to afford the desired compound (250mg,99%).

Synthesis of N,N,N,N=,N=,N=-tetramethyldipalmityl-triethyleneglycoldiamine [compound 24]

Compound 23 (250mg, 0.4mmol) was dissolved in an excess of iodomethane (3ml) and the solution allowed to react overnight at room temperature. The excess reagent was removed under vacuum to produce the the desired tetraamnonium salt (358mg, 99%). This material was formulated with a colipid dioleoylphosphatidylethanolamine (DOPE) as discussed above, and as disclosed in International Application No. PCT/US97/09093, International Publication No. WO 97/42819, at a 1:1.5 lipid/DOPE molar ratio.

As indicated above and as shown specifically in Figure 5, commercially available glycol amine (compound 20), for which n is 2, was reacted with palmitoyl chloride ($R_1 = C_{15}H_{31}$) in the presence of triethylamine to produce compound 21 in a 92% yield. The

resulting compound was reduced with lithium aluminum hydride to produce the secondary amine (compound 22) in an 84% yield. Sequential iodomethylation, followed by free base liberation and methylation of the intermediate tertiary amine (compound 23) produced the desired lipid (compound 24) in a 98% overall yield. Compound 24 was formulated into liposomes in essentially the same manner as described above in connection with Example 19.

TABLE VI
TRANSFECTION OF COS 1 CELLS

Lipid/DOPE (molar ratio)	Liposome Amount (μg)	β-Gal Positive Cells (%)
Compound 24: DOPE, 1:1.5	2 μg	less than 5%
Compound 24: DOPE, 1:1.5	6 μg	10 - 15%
Compound 24: DOPE, 1:1.5	8 μg	50 - 80%
Compound 24: DOPE, 1:1.5	10 μg	15 - 20%

Cells were plated in 35 mm well plates at a density of 1 x 10^5 per well in 1 ml of growth medium. After 24 h (ca 70% confluence), the cells were washed with serum free medium and transfected with 2 μ of plasmid pCMV-β gal. Cells were assayed 48 h after transfection for the β gal gene protein.

TABLE VII
TRANSFECTION OF CHO CELLS

Lipid/DOPE (molar ratio)	DNA/Liposomes Amounts (μg)	β Gal Positive Cells (%)
Compound 24: DOPE, 1:1.5	1:4	less than 10%
Compound 24: DOPE, 1:1.5	2:8	20-25%
Compound 24: DOPE, 1:1.5	3:12	30-70%

Compound 24: DOPE, 1:1.5	4:16	30-35%

Cells were transfected with the plasmid pCMV-β gal in 35 mm well plates at 80% confluence in 1 ml of growth medium. After 24 h, the cells were assayed for the expression of the β gal gene protein.

Example 26

The liposomes produced in accordance with Example 25 were used to transfect COS1 cells and CHO cells following essentially the same procedures described in connection with Examples 20-22 using the amounts of liposomes and slight changes to the procedure as shown in connection with Tables VI and VII respectively.

As shown in Tables VI and VII, as much as 80% of the COS 1 cells and up to 70% of the CHO cells were transfected employing compound 24 as a transfecting agent.

Glyceryl Backbone Embodiments

In further embodiments of the invention, lipids having a glyceryl backbone are provided. These glyceryl backbone based lipids have a dicationic bicyclic triethylene diammonium polar head. The high charge density makes the binding of this class of lipids to DNA molecules stronger than that of the traditional monocationic liposomes for DNA transfection such as DOTMA (Lipofectin reagent™, Life Technologies, Inc.).

These glyceryl backbone based lipids have shown to be very effective transfection reagents with cultured cells, particularly with COS cells.

Scheme V of Figure 5 shows the general synthesis of glyceryl backbone embodiments, which contain the previously unreported triethylenediammonium bicyclic

polar head. As shown in the figure, diol (16) was acylated with palmitoyl chloride (R= $C_{15}H_{31}$) in the presence of triethylamine to afford the diester (17) in a 59% yield. Treatment of the bromide (17) with an excess of the very nucleophilic triethylenediamine (trade name Dabco®) gave the desired lipid (18) in a 44% yield. Further methylation of compound (18) with an excess of iodomethane produced diquaternary amine lipid (19). The corresponding ethers of these class of compounds are made by simply using alkyl iodides or activated esters, such as tosylates or triflates, instead of the acyl halides in the first step. Thereafter, the synthetic route is identical for both types of analogs.

Example 26

Synthesis of 3-bromo-1,2- palmityl propanediol [compound 26]

To a solution of 3-bromo-1,2-propanediol (500 mg, 3.22 mmol) and triethylamine (653 mg, 6.45 mmol) in dichloromethane (20ml) at 0°C under argon was added (dropwise) palmitoyl chloride (1.77g, 6.45 mmol) and the resulting cloudy mixture stirred for 3h at room temperature. Then, a 20% excess of reagents was added and the reaction stirred overnight at room temperature. The reaction was diluted with dichloromethane (50ml) quenched and washed with 10% aqueous sodium bicarbonate (3x 10ml), dried (Na2SO4), filtered, and the solvent removed in vacuo to afford impure desired diester. The crude product was purified by silica gel column chromatography (dichloromethane eluent) to afford pure product (1.2g, 59%)

Synthesis of 1,2-palmityl-3-triethylenediammonium propanediol [compound 27]

A solution of 3-bromo-1,2-palmitylpropanediol (50mg, 0.07 mmol) and

triethylenediamine (Dabco ®, 150mg, 1.33 mmol) in dry dimethyl formamide (3ml) was heated for 12h at 70 °C. The reaction mixture was diluted with dichloromethane (15ml) and washed with water (5x, 5ml). The organic layer was dried (Na_2SO_4), filtered and the solvent removed *in vacuo* to afford the desired material (26mg, 44%). This material was formulated with a colipid dioleoyl phosphatidyl ethanolamine (DOPE) at a 1:1.5., Lipid:DOPE, respectively.

As indicated above and as shown in Figure 6, compound 26 is produced by acylating the starting diol with palmitoyl chloride in the presence of triethylamine to produce the diester in a 59% yield. The diester is treated with an excess of the highly nucleophilic triethylene diamine (Dabco ®) which gives the desired lipid (compound 27) in a 44% yield. Further methylation with an excess of iodomethane produced a diquaternary amine lipid (compound 28). The corresponding ethers are made by using alkyl iodides (e.g. methyl iodide or activated esters (e.g. tosylates or triflates) instead of the acyl halides in the first step.

Example 27

Liposomes containing lipids produced in accordance with the present invention and particular compounds 6d, 18 and 24 were employed to transfect a variety of cell types as listed in Table VIII.

TABLE VIII
DNA or RNATRANSFECTION OF OTHER CELL TYPES
WITH LIPOSOMES MADE WITH LIPIDS 6d, 18 and 24

Lipid	Cell type, nature	Positive cells
6d	PC12, Phaeochromocytpoma cells, secondary	45-50%
6d	C2C12, mouse muscle myoblast, secondary	15-20%
6d	quail myoblasts, primary	50-80%
6d	rat fibroblats, primary	35-40%
6d	rat cardiomyocytes, primary	20-30%
6d	rat retinal neurons, primary	20-23%
18	COS-1, kidney monkey , secondary	30-35%
24	T-24, human bladder carcinoma, secondary	60-80%
24	Drosophila Schneider, insect, secondary	60-90%
24	SF-9, ovary fall army worm, secondary	30-40%
24	rat smooth muscle, primary	35-40%
24	RNA Transfection in 293 cells, human kidney	40-80%

Table VIII shows remarkable transfection efficiencies for liposomes derived from compounds (6d) and (24). Especially, compound (6d) has proven to be an outstanding reagent for the transfection of transfection-refractory or difficult-to-transfect cells types such as primary neurones and primary quail myoblasts, respectively. Normally, liposome reagents of the previous art were considered superior if transfection efficiencies approaching 5-10% were obtained with primary cells. Compound (6d) has produced transfection efficiencies for the latter type of cells of up to 80%. On the other hand, compound (24) gives very good transfection efficiencies with primary rat smooth muscle cells and superb efficiencies with difficult-to-transfect insect cells. Moreover, this compound works, without diminished activity, in the presence of 10% serum. This characteristic is a desirable one for the practice of gene therapy since the cells in the human body are in permanent contact with pure serum, so any liposome reagent that has little or

no activity in the presence of serum has a limited use in this therapeutic arena. Finally, compound (24) is also an outstanding reagent for the transfection of RNA into human kidney cells.

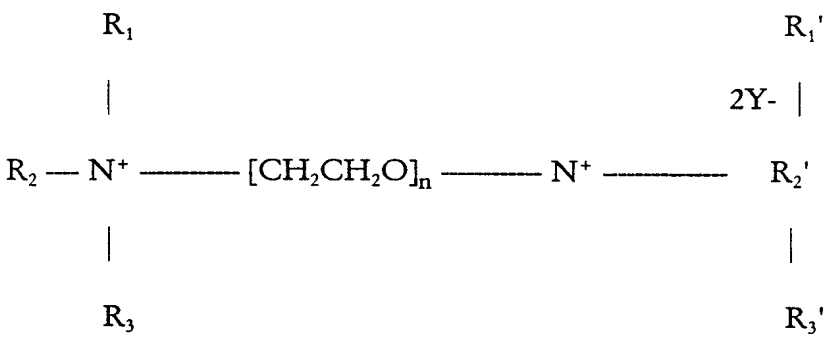
Thus, as shown in Table VIII, the lipids of the present invention provided significant assistance in the transfection of a variety of cell types and in several cases resulted in positive transfection rates of as high as 80 or 90%.

Having described the inventions with regard to specific embodiments, it is to be understood that the description is not meant as a limitation since further variations or modifications may be apparent or may suggest themselves to those skilled in the art. It is intended that the present application cover all variations and modifications of the inventions as fall within the scope of the appended claims.

Claims

What is claimed is:

1 A composition comprising the mixture of a compound having the structure:



R_1, R_1' = independently: linear alkyl C_1-C_{18}

R_2, R_2' = independently: linear alkyl C_1 -C_{18}, linear alkyl C_1 - C_4, guanidium or amidinium, aminopropyl, 4-a butyrylamidopropyl, cyanoethyl

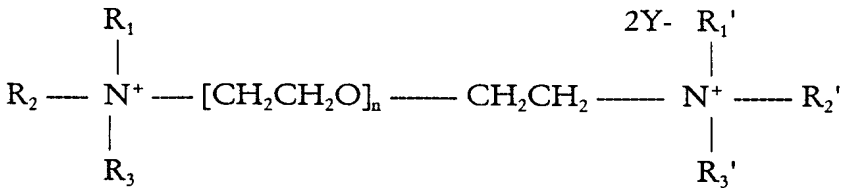
R_3, R_3' = independently: linear alkyl C_1-C_6, acetoxyethyl, cyanoethyl.

Y = a pharmaceutically acceptable anion

n= 2-50, or higher

and a member of the class consisting of nucleic acids, oligonucleotides, mononucleotides, polypeptides and proteins.

2. A compound having the structure:



R_1, R_1' = independently: linear alkyl C_1-C_{18}

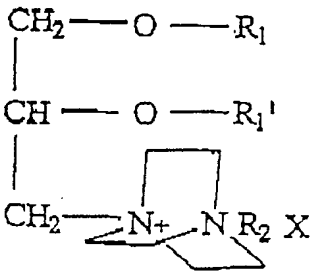
R_2, R_2' = independently: linear alkyl C_1-C_{18}, linear alkyl C1-C4, guanidium or amidinium, aminopropyl, 4-a butyrylamidopropyl, cyanoethyl

R_3, R_3' = independently: linear alkyl C_1-C_6, acetoxyethyl, cyanoethyl.

Y = a pharmaceutically acceptable anion

n = 2-10.

3. A composition comprising the mixture of a compound having the structure:



R_1, R_1' = C_1 to C_{20} alkyl group, C_1 to C_{20} acyl group (Independently;

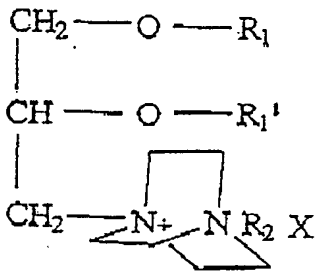
R_2 = Electron pair; C_1 to C_6 alkyl group; CH_2CONH_2; CH_2CO_2Methyl, Ethyl or H, CH_2CN; amino C_2 to C_4 alkyl; hydroxy C_2 to C_4 alkyl; polyamino alkyl

X = pharmaceutically acceptable anion(s),

and a member of the class consisting of nucleic acids oligonucleotides, mononucleotides, polypeptides, and proteins

What is Claimed:

4. A ~~composition comprising the mixture of a compound~~ compound having the structure:



R_1, R_1' = (independently:) C_1 to C_{20} alkyl group, C_1 to C_{20} acyl group

R_2 = Electron pair; C_1 to C_6 alkyl group; CH_2CONH_2; CH_2CO_2Methyl, Ethyl or H, CH_2CN; amino C_2 to C_4 alkyl; hydroxy C_2 to C_4 alkyl; polyamino alkyl

X = pharmaceutically acceptable anion(s)

5. A liposome comprising the compound of claim 2.

6. A liposome comprising the compound of claim 4.

7. The compound of claim 2 which is N, N, N, N, N', N', N', N' - tetramethyldipalmityl - triethyleneglycoldiamine.

8. A composition comprising the compound of claim 7 and a member of a class consisting of nucleic acids, oligonucleotides, mononucleotides, polypeptides, and proteins.

9. The compound of claim 4 which is 1, 2 - palmityl - 3- triethylenediammonium propanediol or alkyhalide derivatives thereof.

10. A composition comprising the compound of claim 9 and a member of a class consisting of nucleic acids, oligonucleotides, mononucleotides, polypeptides, and proteins.

11. The composition of claim 1, further comprising a colipid.

12. The composition of claim 3, further comprising a colipid.

13. The composition of claim 8, further comprising a colipid.

14. The composition of claim 10, further comprising a colipid.

15. A method of delivering a negatively charged macromolecule to a cell comprising delivering to said cell the composition of claim 1.

16. A method of delivering a negatively charged macromolecule to a cell comprising delivering to said cell the composition of claim 3.

17. The method of claim 15, wherein the composition further comprises a colipid.

18. The method of claim 16, wherein the composition further comprises a colipid.

SCHEME I

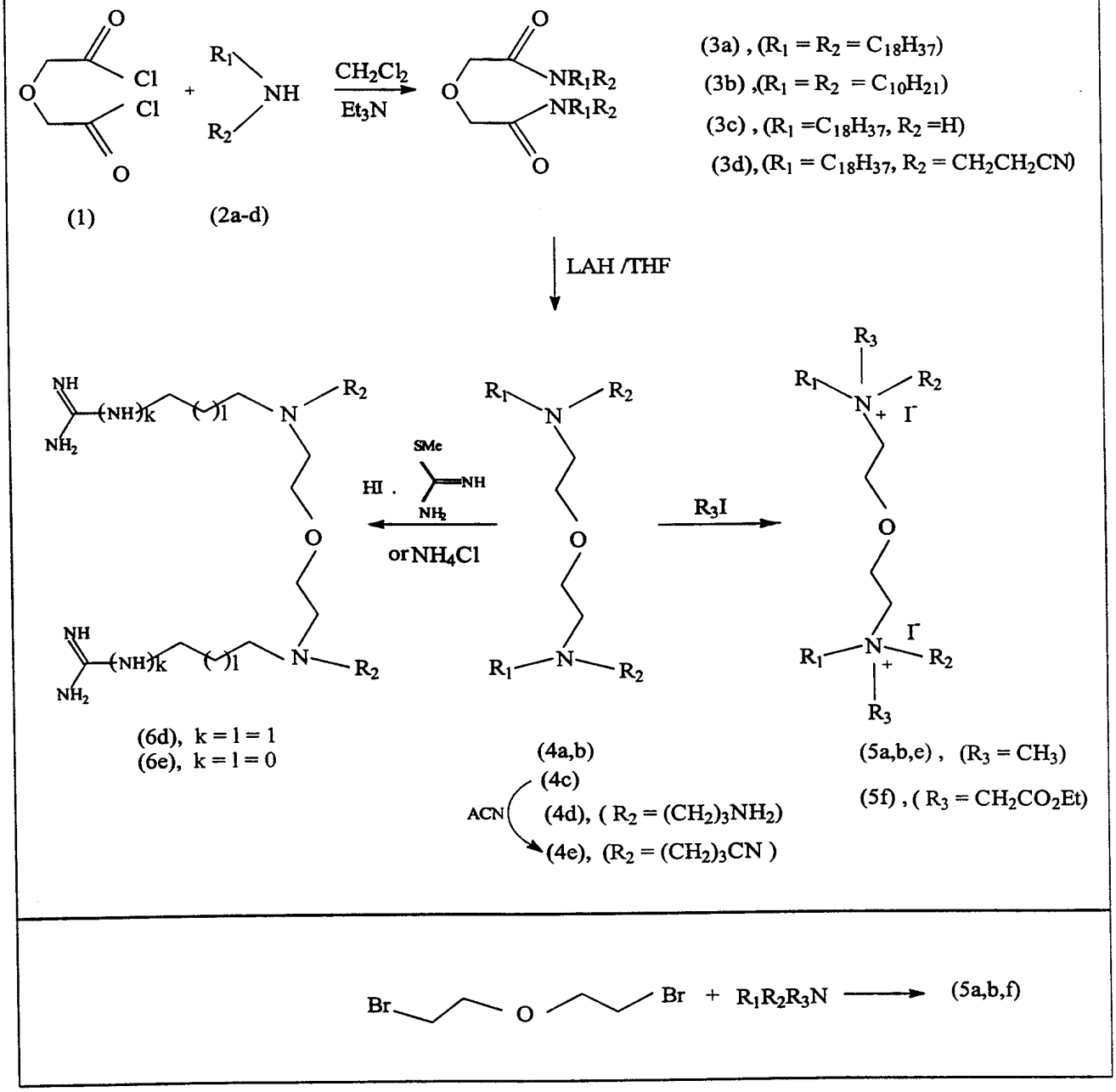


FIGURE 1

SCHEME II

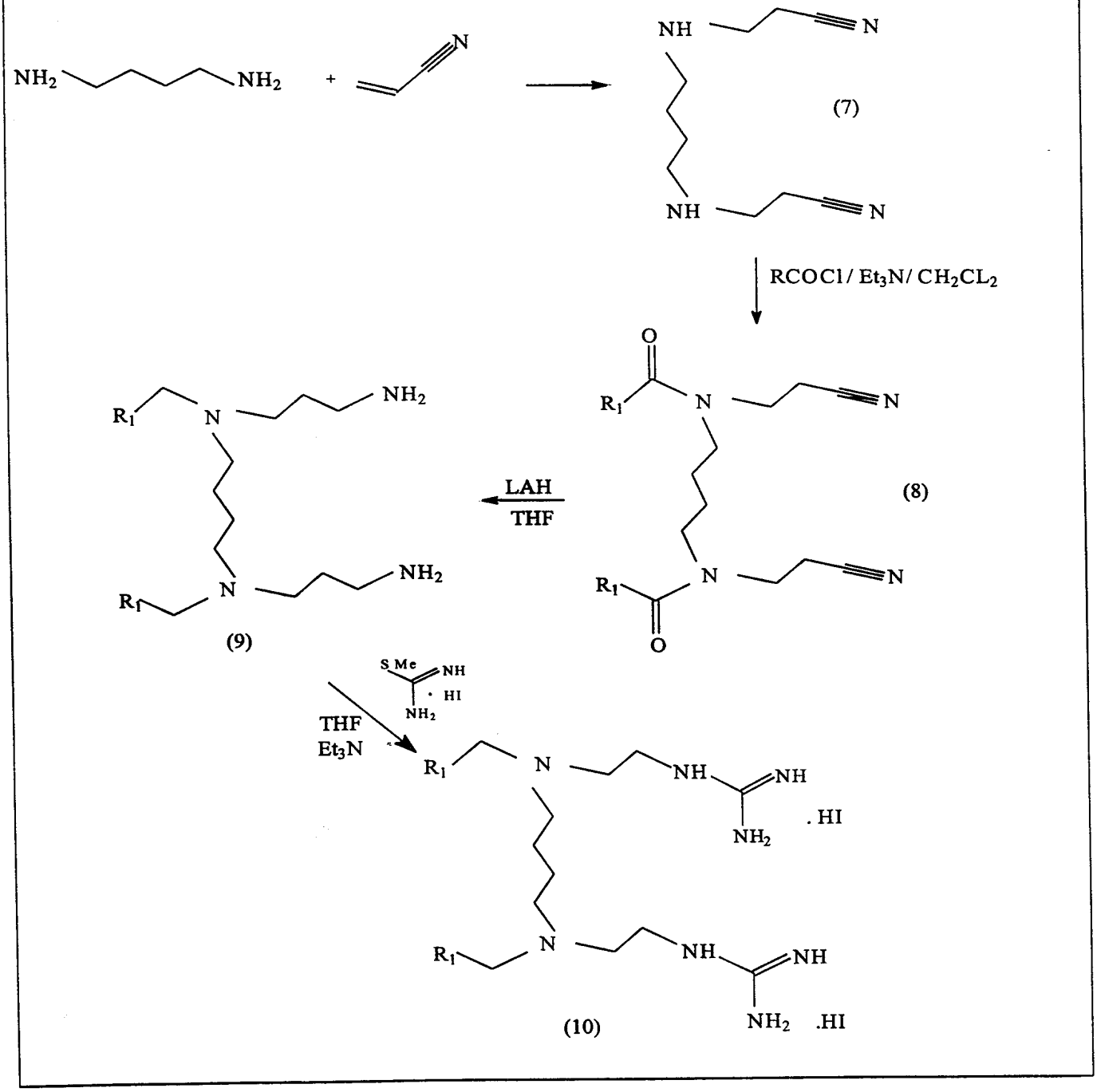


Fig-2

SCHEME III

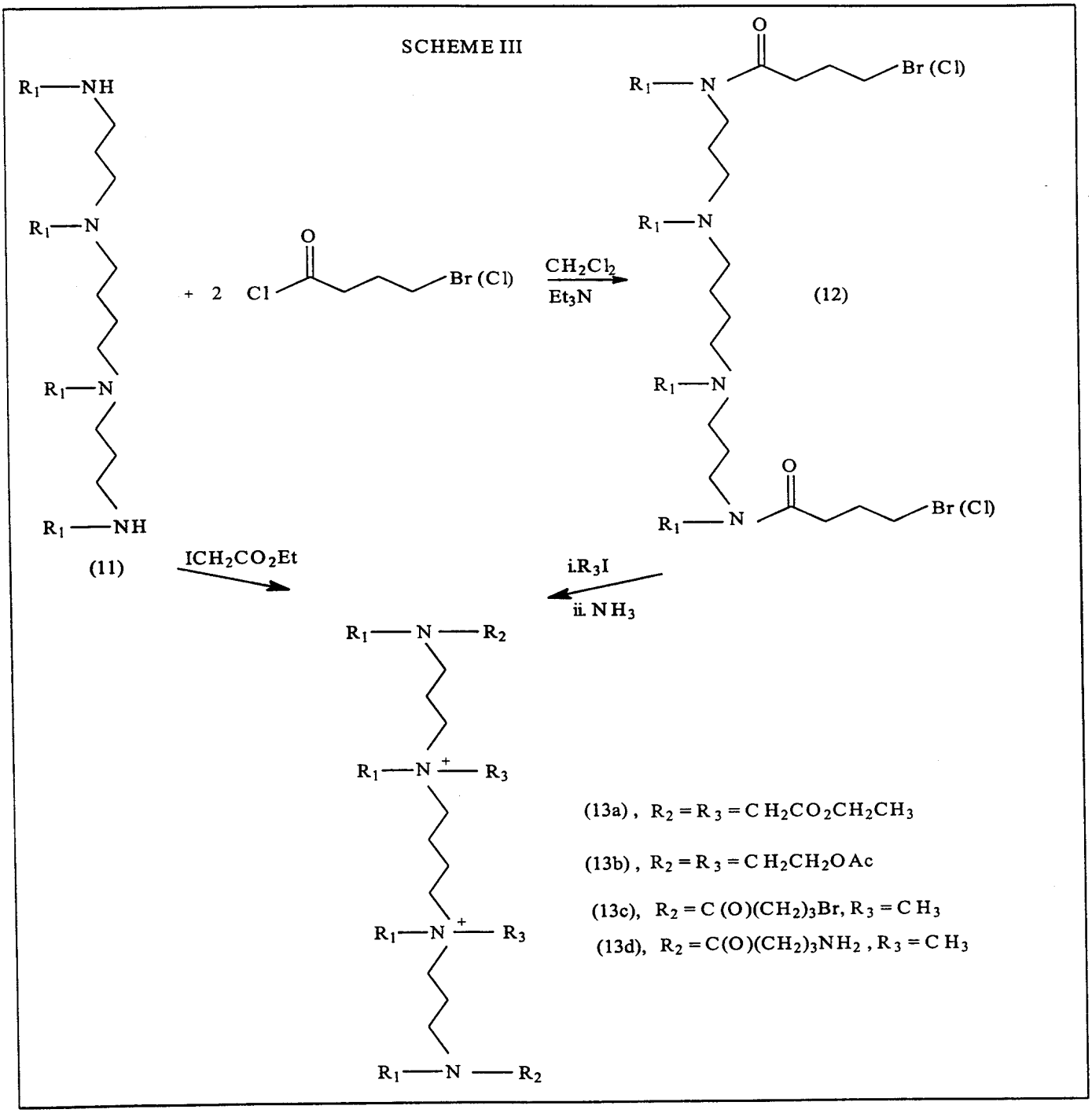


Fig. 3

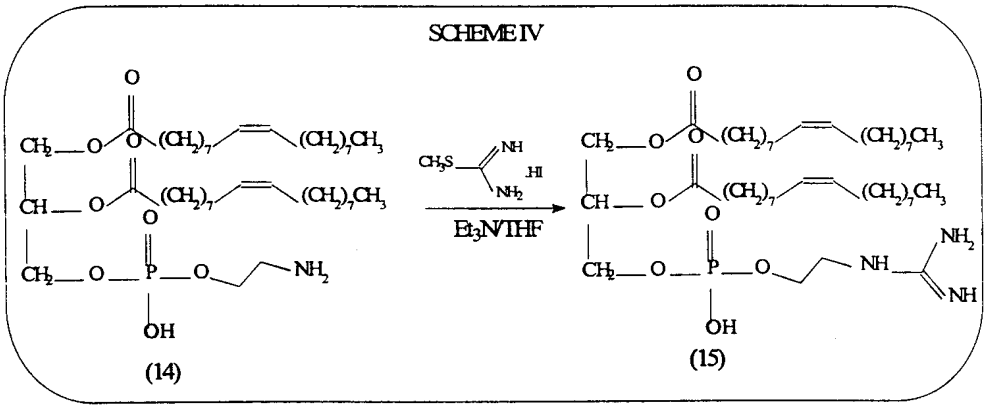
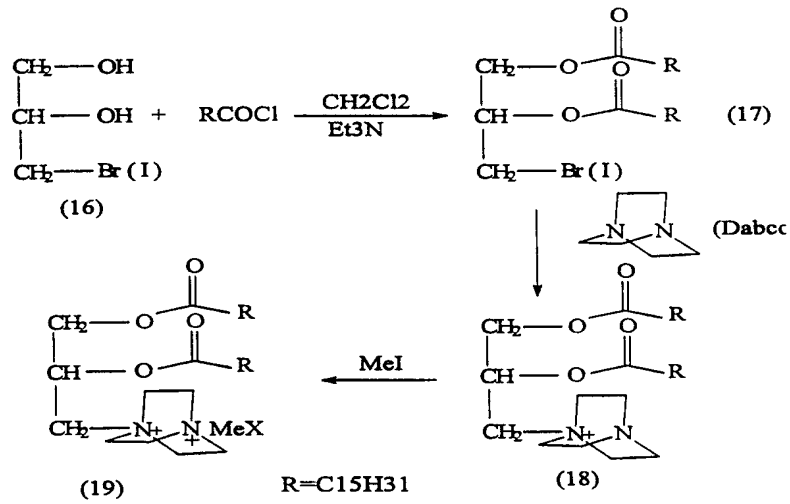


Figure 4

SCHEME V (Glyceryl back. Embodiment

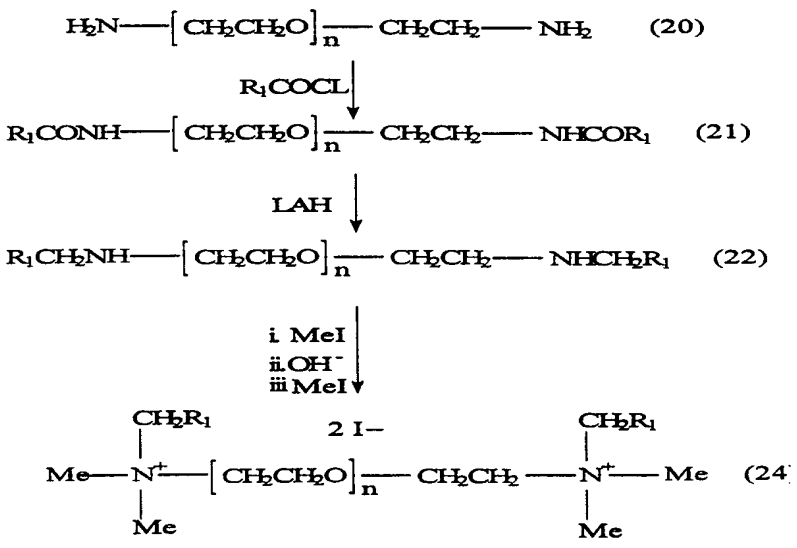


Scheme V shows the general synthesis of glyceryl backbone embodiments, which contain the previously unreported triethylenediammonium bicyclic polar head.

Thus, diol (16) was acylated with palmitoyl chloride (R= $C_{15}H_{31}$) in the presence of triethylamine to afford the diester (17) in a 59% yield. Treatment of the bromide (17) with an excess of the very nucleophilic triethylenediamine (trade name Dabco®) gave the desired lipid (18) in a 44% yield. Further methylation of compound (18) with an excess of iodomethane produced diquaternary amine lipid (19). The corresponding ethers of these class of compounds are made by simply using alkyl iodides or activated esters, such as tosylates or triflates, instead of the acyl halides in the first step. Thereafter, the synthetic route is identical for both types of analogs.

Fig. 5

SCHEME VI (Polyether back. Embc

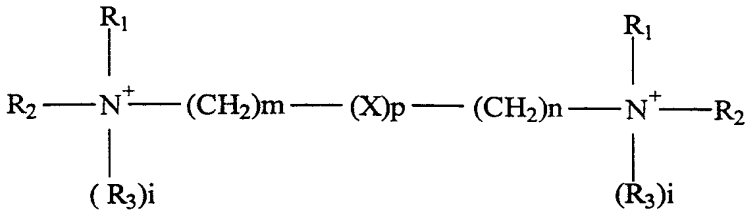


Scheme VI depicts the general synthetic route to prepare the polyether backbone embodiments of the present invention.

Reacting the commercially available triethylene glycol diamine (20), for which n=2, with palmitoyl chloride ($R_1 = C_{15}H_{31}$) in the presence of triethylamine, compound (21) was obtained in a 92 % yield. Lithium aluminun hydride reduction of diamide (21) afforded the correspoding secondary diamine (22) in 84 % yield. Sequential iodomethane methylation of (22), followed by free base liberation and methylation of the intermediate tertiary amine (23) afforded the desired lipid (24) in 98%overall yield. Lipid (24) was formulated into liposomes as per example 19 and successfully tested in several cell types.

Fig. 6

Formula I



X = O, S, S(O), NH
m,n = 1, 2, 3
p = 0 ,1
i = 0, 1

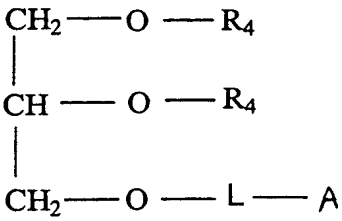
R_1 = linear alkyl C_1 - C_{18}
R_2 = linear alkyl C_1 - C_{18} , linear alkyl C_1 - C_4 guanidinium or amidinium, aminopropyl, 4-a butyrylamidopropyl, cyanoethyl
R_3 = linear alkyl C_1 - C_6, acetoxyethyl, CH_2-$CO_2CH_2CH_3$

X = CH_2 when R_3 is not a linear alkyl C_1-C_{18}

Figure 7

FIGURE 7

Formula 2



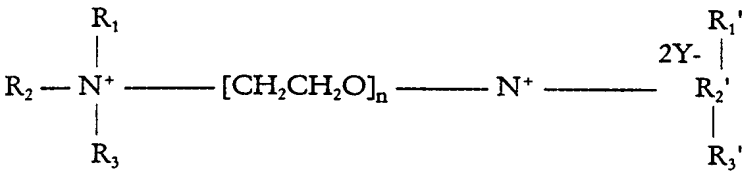
R_4, R_4', = Independently: linear $(CO)C_6$-C_{20} saturated or unsaturated hydrocarbons, C_6-C_{20} saturated or unsaturated hydrocarbons.

L = 2-10 atoms linker

A = Guanidinium; amidinium; guanidylated polyamines.

Figure 8

Formula 3 - polyether embodiments



R_1, R_1' = independently: linear alkyl C_1-C_{18}

R_2, R_2' = independently: linear alkyl C_1 -C_{18}, linear alkyl C_1 - C_4, guanidium or amidinium, aminopropyl, 4-a butyrylamidopropyl, cyanoethyl

R_3, R_3' = independently: linear alkyl C_1-C_6, acetoxyethyl, cyanoethyl.

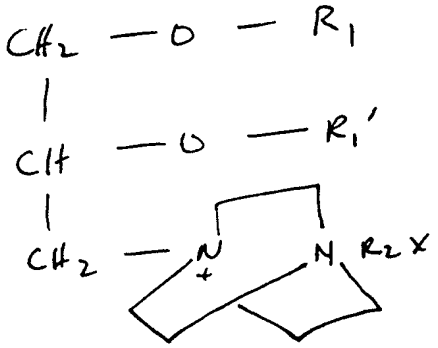
Y = a pharmaceutically acceptable anion

n= 2-50, or higher

Figure 9

FIGURE 10

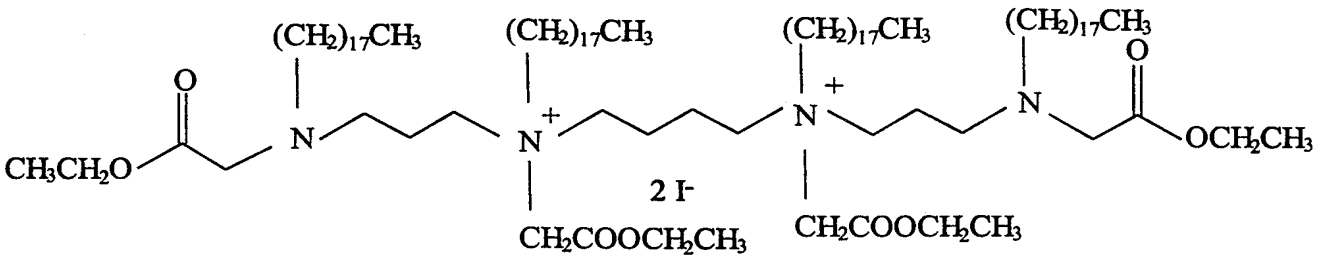
Formula 4 - Glyceryl backbone embodiments



R_1, R_1' = independently C_1 to C_{20} alkyl group, C_1 to C_{20} acyl group

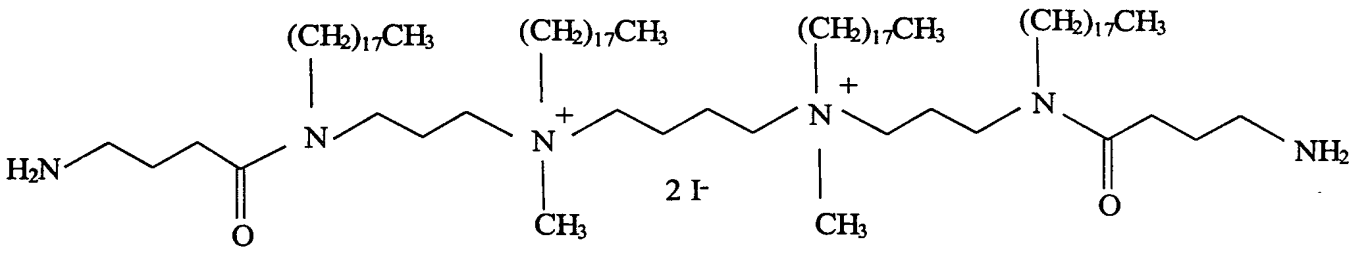
R_2 = electron pair; C_1 to C_6 alkyl group; CH_2CONH_2; CH_2CO_2 methyl, Ethyl or H, CH_2CN; amino C_2 to C_4 alkyl; hydroxy C_2 to C_4 alkyl; polyamino alkyl.

X = pharmaceutically acceptable anion(s).



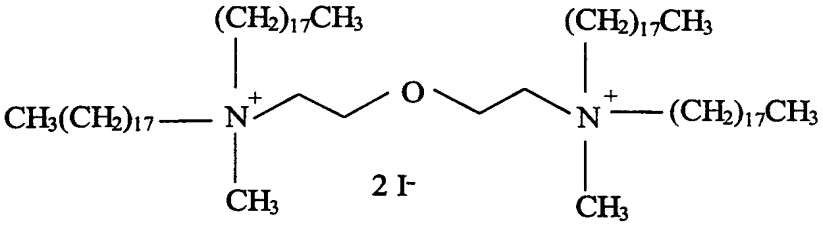
Compound 13a

Figure 11



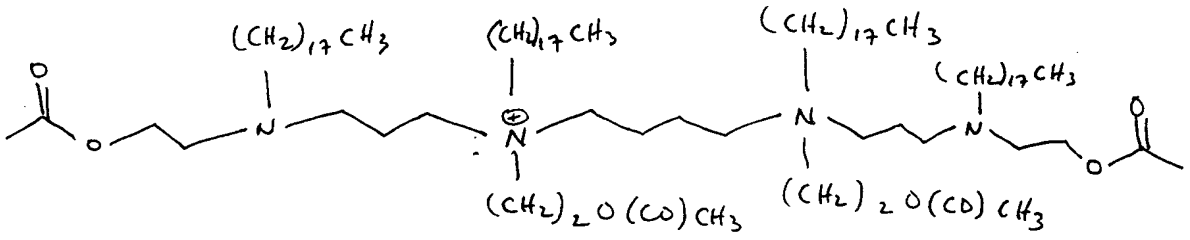
Compound 13d

Figure 12



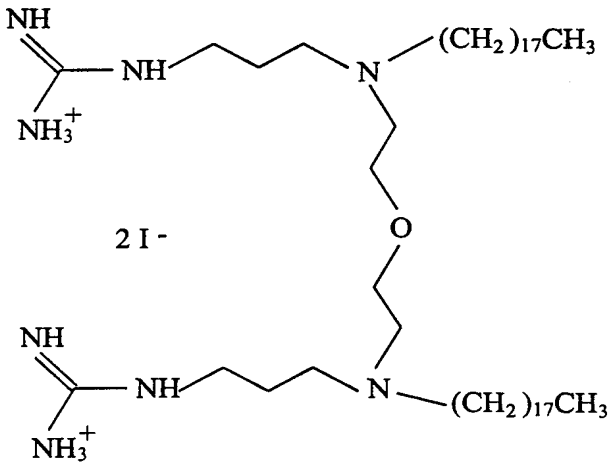
Compound 5A

Figure 13



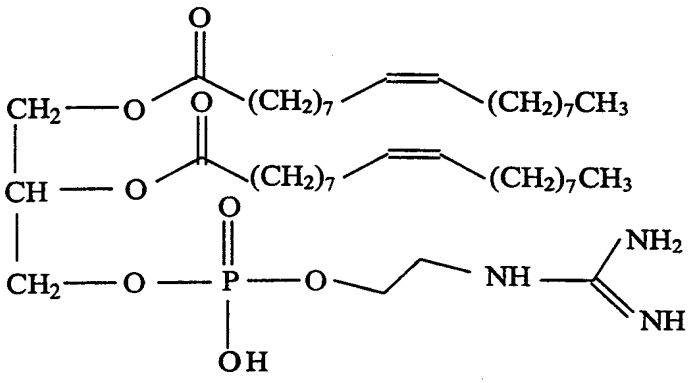
Compound 13b

Figure 14



Compound 6d

Figure 15



Compound 15

Figure 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19629

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07C 19/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST online

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,897,355 A (EPPSTEIN et al) 30 January 1990, cols. 29-60.	1-8
Y	US 5,171,678 A (BEHR et al.) 15 December 1992, cols. 1-6.	1-18
Y	US 5,334,761 A (GEBEYEHU et al.) 02 August 1994, cols. 5-20.	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search 04 DECEMBER 1999	Date of mailing of the international search report 03 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JAMES O. WILSON Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19629

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/18, 19, 20, 44, 47, 48, 51
530/300, 350
564/197, 292, 504

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

514/18, 19, 20, 44, 47, 48, 51
530/300, 350
564/197, 292, 504